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Dead or dying: the importance of time in cytotoxicity assays using arsenite as an example

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Abstract

Arsenite is a toxicant and environmental pollutant associated with multisite neoplasias and other health effects. The wide range of doses used and the claims that some high doses are "not toxic" in some assays have confounded studies on its mechanism of action. The purpose of this study is to determine whether the treatment time and particularly the duration between treatment and assay are important factors in assessing arsenite toxicity. We compared three commonly used assays: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red (NR), and clonal survival, using human osteogenic sarcoma cell line U-2OS. Results from the assays were well correlated only when the factor of time was taken into account. In both the MTT and NR assays, exposure to arsenite for 24 h induced much less toxicity than exposure for 48 or 72 h, which gave similar results. In contrast, results in clonal survival assays showed only a small difference between 24-h exposure and longer exposure times. Arsenite demonstrated delayed cytotoxicity, killing the cells even after its removal from the medium in NR assay. Apoptosis was assessed by TUNEL staining and caspase-3 activation. After treatment for 24 h with 0.1 and 1 μM arsenite, no apoptosis was seen. However, after an additional 24 h in arsenite-free medium, a small amount of apoptosis could be detected, and much more apoptosis was seen after 48 h. In contrast, 10 μM arsenite triggered rapid necrosis and failed to activate caspase 3 or cause TUNEL staining. We also confirmed previous reports that exposure to low concentrations of arsenite caused transient stimulation of cell growth. Our finding of delayed toxicity by arsenite suggests that to avoid underestimation of toxicity, the duration between treatment and assay should be taken into account in choosing appropriate doses for arsenite as well as for other toxicants that may show similar delayed toxicity. The NR and MTT assays should be performed only after an interval of at least 48 h after a 24-h exposure to arsenite.

Keywords: Arsenite; Cytotoxicity; Cell viability; Human osteosarcoma cells; Proliferation; Apoptosis; Caspase 3; Necrosis

Introduction

Cell viability determination is crucial for evaluating mutagenesis, malignant transformation, apoptosis, cellular pathology, and pharmaceutical toxicity. It is also of importance in elucidating mechanisms of carcinogenesis because tumors will arise only from living cells. Signaling and other changes in dying cells are thus meaningless in carcinogenesis research, and are of use only for studying mechanisms of cell death.

A toxic compound may initiate two distinct events: apoptosis or necrosis, two forms of cell death with clearly distinguishing morphological and biochemical features (Wyllie et al., 1980). Typical features of apoptosis are fragmented nuclei with condensed chromatin, shrunken cytoplasm within a nearly intact plasma membrane (Kerr et al., 1972), and organized DNA degradation (Duke et al., 1983; Wyllie, 1980). Loss of membrane integrity and leakage of cellular components are characteristic of necrosis (Wyllie et al., 1980). The same agent can cause both apoptosis and necrosis. For example, <1 μM cadmium chloride induced apoptosis, whereas higher concentrations caused necrotic death without initiation of apoptotic pathways (Lopez et al., 2003). Thus, classical apoptosis and necrosis represent only the extreme ends of a wide range of possible morphological and biochemical deaths and can occur simultaneously in tissues and

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cell cultures exposed to the same stimulus (Nicotera et al., 1999). Apoptosis may eventually progress to secondary necrosis when dying cells fail to be removed by heterophagy (Kroemer et al., 1998). Possible mechanistic explanations for the switch between apoptosis and necrosis have been reviewed (McConkey, 1998; Raff, 1998).

The clonal survival assay has been considered the gold standard for determining viability of cells in vitro because it is highly stringent. Some technical problems, such as low plating efficiencies, clumping artifacts, and assay duration, may limit the applicability of clonogenic assays of some cells. To overcome these limitations, some short-term assays based on the measurement of parameters that indicate the number of dead cells, the number of live cells, or the mechanism of cell death have been developed.

Vital dyes such as trypan blue or fast green are usually excluded from viable cells but can enter through damaged cell membranes to stain the cytoplasm, and they have long been used as indicators of nonviable cells. Trypan blue was used to study the cytotoxicity of antitumor drugs in a variety of tumors, and a good correlation was found between the in vitro responsiveness and the in vivo chemotherapy results in both animals and humans (Durkin et al., 1979). Similar results were found using fast green as an in vitro indicator of cell death (Weisenthal et al., 1983). The limitation of vital dye exclusion tests is that these assays measure dead necrotic cells with damaged membranes and may not measure dying or apoptotic cells with intact membranes.

Measuring the leakage of components out of the cytoplasm through damaged cell membranes can also indicate loss of membrane integrity. Decker and Lohmann-Matthes (1988) described a simple method of measuring lactate dehydrogenase (LDH) release from lysed tumor cells. LDH is a stable enzyme normally found in the cytosol of all cells, and is rapidly released into the supernatant following cell death. Its activity in the medium is a marker of necrosis. Measuring enzyme release from cells has the same limitation as vital dye exclusion because they both measure necrotic cells with damaged membranes. However, the LDH release assay is an important tool for assessing necrosis.

Although LDH release is considered a marker for necrosis, caspase-3 activation is frequently used as a marker for apoptosis (Nicholson et al., 1995). Activation of the caspase cascade is an integral event in the apoptotic pathway (Alnemri et al., 1996; Atkinson et al., 1998). Caspase 3 is part of the caspase cascade that triggers the cleavage of numerous proteins leading to the ordered breakdown of the cell (Tewari et al., 1995).

Viable cells can be measured with colorimetric assays utilizing a substrate whose color is modified by living cells, but not by dead cells. Mosmann (1983) developed a rapid colorimetric assay based on the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which is cleaved and converted to a blue formazan by the mitochondrial dehydrogenases in active mitochondria. Good correlation was observed between MTT and clonogenic assays for drug exposures in some cell lines (Carmichael et al., 1987).

Another rapid colorimetric test is based on the uptake of the cationic supravital dye neutral red (NR) (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) into viable cells, where it accumulates in lysosomes, binding electrostatically to anionic sites in the lysosomal matrix (Bulychev et al., 1978). Damage to the cell surface or sensitive lysosomal membranes decreases the uptake and binding of NR (Lullmann-Rauch, 1979), making it possible to differentiate between viable intact cells and dead/damaged cells. The NR assay was developed and successfully used to evaluate the cytotoxic effect of metal salts in mouse BALB/c 3T3 fibroblasts (Borenfreund and Puerner, 1985, 1986).

The concentration of test agent and duration of exposure are two factors in the subsequent development of in vitro cytotoxicity. In most studies, cell viability is measured soon after toxicant exposure. Relatively high concentrations are thus required to obtain acute cytotoxic effects. In the case of arsenic compounds, we were concerned that a fraction of cells will eventually die after sodium arsenite ($NaAsO_2$) treatment, and that many commonly used assays underestimate dying cells. Arsenic is an environmental contaminant, and arsenite is the most likely environmental carcinogenic form of arsenic (International Agency for Research on Cancer, 1980). Previously, this laboratory demonstrated that arsenite in drinking water enhances ultraviolet irradiation-induced skin carcinoma in the mouse (Rossman et al., 2001) and causes delayed transformation and mutagenesis in human osteogenic sarcoma (HOS) cells (Mure et al., 2003). HOS cells contain mutant p53 genes (Romano et al., 1989). It was reported that p53-deficient cells are hypersensitive to arsenite-induced cytotoxicity (States et al., 2002). However, another study showed that p53 is not involved in arsenite-induced apoptosis (Huang et al., 1999). Nevertheless, for this study, we use human osteogenic sarcoma cell line U-2OS that has wild-type p53 genes and a level of p53 protein comparable to the level in normal diploid fibroblasts (Diller et al., 1990).

Materials and methods

Chemicals and reagents. Sodium arsenite, MTT, trypan blue, pyruvate, NADH (preweighed vials), oxamate, and hematoxylin were purchased from Sigma (St. Louis, MO). NR was purchased from Fisher Scientific (Fair Lawn, NJ).

Cell culture. The human osteogenic sarcoma cell line U-2OS (American Type Culture Collection, Rockville, MD) was grown in DMEM (Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum (Omega Scientific, Inc., Tarzana, CA) at 37 °C in 5% CO_2 atmosphere. Sodium arsenite (freshly prepared stock solution) was added to the attached cells at concentrations from 0.01 to 10 μM for the times indicated.